Studies *in vivo* of the tissue uptake, cellular distribution and catabolic turnover of exogenous glucocerebrosidase in rat

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The kinetics of plasma clearance of highly purified human placental glucocerebrosidase in rats were biphasic with 75% of the infused dose showing a rapid clearance ($t_1 = 11$ min) and the remaining 25% a considerably lower rate ($t_1 = 60$ min). The majority of the enzyme (60%) was taken up by the liver. Although saturation kinetics for the clearance or uptake were not observed, the very high hepatic endocytic index ($217 \mu\text{l}/\text{min}$) of glucocerebrosidase uptake indicated that liver uptake was mediated by an adsorptive endocytic process. Analysis of the cellular distribution of recovered glucocerebrosidase revealed predominantly parenchymal cell uptake with 38% of the exogenous enzyme in hepatocytes and only 2% in sinusoidal cells. High-mannose glycoproteins blocked hepatocyte and sinusoidal cell uptake of glucocerebrosidase equally. Kinetic experiments failed to demonstrate a transfer or shuttle of exogenous glucocerebrosidase from sinusoidal cells to hepatocytes. The possibility was raised that uptake of enzyme by the liver may be mediated by a common receptor that functions in both hepatocytes and sinusoidal cells. The catabolic turnover of exogenous glucocerebrosidase in rat liver was biphasic and the rate of decline was similar in hepatocytes and sinusoidal cells.

Intravenous administration of glucocerebrosidase in humans has served as a model for evaluating the potential of enzyme replacement for the treatment of metabolic disorders. Several characteristics of the enzyme make it particularly suitable for replacement trials. The lysosomal localization of the enzyme (Weinreb *et al.*, 1968), its deficiency as manifested by metabolite accumulation primarily within the reticuloendothelial system (Brady, 1966) and the relative ease and efficiency of the enzyme's isolation from human tissue (Furbish *et al.*, 1977) have encouraged its application in such studies. Early clinical trials of glucocerebrosidase administration to patients with Gaucher's disease, who are deficient in this enzyme, were encouraging (Brady *et al.*, 1974). After infusion of highly purified human placental glucocerebrosidase (Pentchev *et al.*, 1973) a decrease in elevated glucocerebroside in the blood and liver of patients was observed. In subsequent

trials with enzyme isolated by a different procedure involving effective removal of lipid material by butanol extraction (Furbish *et al.*, 1977) the results were not as conclusive (Brady *et al.*, 1980a). In some patients a notable decrease in hepatic and blood glycolipid concentrations was found, whereas in other patients no biochemical consequence of the enzyme treatments was detected (Brady *et al.*, 1980b). In an attempt to gain more insight into the mechanisms of such corrective approaches, the present studies were designed to characterize the behaviour of exogenous glucocerebrosidase *in vivo*. The studies revealed several relevant phenomena that future research efforts will have to address themselves in order to develop more effective enzyme-replacement strategies for Gaucher's disease.

Experimental

Materials

Human placental glucocerebrosidase was purified and prepared as previously described (Furbish *et al.*,

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1977). The specific activity of the enzyme was 1×10^6 nmol/h per mg of protein. Heat-denatured human serum albumin was prepared as described by Ho & Wagner (1963). Fetuin (GIBCO) was dissolved in 0.05 M- H_2SO_4 and heated at 80°C for 1 h to prepare asialofetuin, as previously described (Spiro, 1966). Terminal galactose residues exposed in the fetuin after hydrolysis were measured by galactose oxidase to be 10 nmol of galactose/nmol of fetuin. Human lactoferrin was a gift from Dr. R. L. Hill, Duke University, Durham, NC, U.S.A. *Escherichia coli* alkaline phosphatase, ovalbumin, horseradish peroxidase, invertase, mannan and metrizamide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (type III) was obtained from Worthington Biochemical Co., Freehold, NJ, U.S.A. Pronase was purchased from Calbiochem-Behring Corp., La Jolla, CA, U.S.A.

Methods

Enzyme labelling by radioiodination. Glucocerebrosidase was shown to be extremely sensitive to oxidizing agents so that even the gentle oxidizing conditions of the lactoperoxidase procedure (Kusiak & Barranger, 1979) caused complete inactivation of catalytic activity. An alternative method of radioiodination involving the Bolton & Hunter (1973) reagent avoided this difficulty. For this, 3 vol. of ice-cold ethanol were added to 100 μl of highly purified glucocerebrosidase (3×10^6 units/ml; 3 mg of protein/ml) in 0.1 M-sodium citrate buffer (pH 5.0)/1 mM-2-mercaptoethanol/5 mM-EDTA to precipitate the enzyme. The suspension was centrifuged at 10000 g for 30 min and the protein pellet was redissolved in 50 μl of ice-cold 0.1 M-borate buffer, pH 8.0. The solution was added to 1 mCi of dried Bolton-Hunter ester reagent (New England Nuclear) and kept at 0°C with frequent mixing for 45 min. The reaction was terminated by addition of 100 μl of 0.2 M-glycine in 0.1 M-borate buffer, pH 8.0. Unbound label was separated by passing the reaction mixture through PD-10 columns (5.5 cm $\times$ 1.5 cm) of Sephadex G-25 M (Pharmacia) equilibrated with 0.9% NaCl. The void volume was collected and dialysed overnight against saline. The catalytic activity of the labelled enzyme was assayed with 4-methylumbelliferyl β -D-glucopyranoside as described by Dale *et al.* (1979). Typical preparations retained 85% of the enzymic activity and contained 1×10^9 d.p.m./mg of protein, of which 95% was trichloroacetic acid-precipitable. ^{125}I material was measured with a Beckman Gamma 400 counter.

Enzyme labelling with [^3H]raffinose. [^3H]-Raffinose was coupled to protein as described previously (VanZile *et al.*, 1979). Briefly, [^3H]-raffinose (New England Nuclear; sp. radioactivity 1.5 Ci/mmol) was converted into raffinialdehyde by using galactose oxidase. [^3H]Raffinialdehyde

(100 nmol) was added to 10 nmol of glucocerebrosidase (600 μg of protein) and 2 μmol of NaBH_3CN (Sigma), and the reaction mixture was left at room temperature overnight. Labelled enzyme was separated from unchanged raffinialdehyde by gel filtration as described for the radioiodinated protein and the final specific radioactivity was 16×10^6 d.p.m./mg of protein. The recovery of enzymic activity was 91%. ^3H -labelled material was measured by liquid-scintillation counting using Bio-fluor (New England Nuclear).

Isolation of liver hepatocytes and sinusoidal cells. The method described by Steer & Clarenburg (1979) for isolation of rat liver cells was employed with certain modifications. The livers of female Sprague-Dawley rats (200–250 g) were washed free of blood by inserting a cannula in the portal vein, cutting the inferior vena cava and flushing the liver with Ca^{2+} -free Krebs-Henseleit buffer equilibrated with O_2/CO_2 (19:1) at 37°C . The bleached livers were carefully transferred to a perfusion-aeration chamber (MAR Corp., Clearwater, FL, U.S.A.) connected to a peristaltic pump. The liver was perfused at 37°C with the above buffer, which also contained 0.03% collagenase, and aerated by gently bubbling O_2/CO_2 (19:1) into the recycled perfusate collected in a chamber below the draining liver. After perfusion for 20 min at a flow rate of 25 ml/min, the softened liver was freed of its capsule and gently scraped with a rubber 'policeman' in 100 ml of gassed Krebs-Henseleit buffer containing Ca^{2+} . The resulting suspension was then filtered twice through eight layers of cheesecloth and the filtrate was left at 4°C for 7 min. The supernatant consisting mainly of sinusoidal cells was pipetted off and set aside. The sedimented hepatocytes were washed twice by gravity with 80 ml of normal gassed Krebs-Henseleit buffer containing 1.3% (w/v) bovine serum albumin. The crude sinusoidal cell preparation was centrifuged at 60 g for 2 min to remove additional hepatocyte contamination and 25 ml samples of the supernatant were layered on 13 ml of ice-cold 16% (w/v) metrizamide in 8.9 cm $\times$ 2.5 cm centrifuge tubes fitted for the Beckman SW-27 rotor. The solutions were centrifuged at 3000 g for 45 min at 20°C . The sinusoidal cells, consisting of a mixture of endothelial and Kupffer cells, collected at the interface of metrizamide and were removed with a pasteur pipette. The cell suspension (10–15 ml) was diluted with gassed Krebs-Henseleit buffer and 1.3% albumin to 40 ml and centrifuged at 500 g for 10 min. The cell pellet was resuspended in 4 ml of the above buffer. Portions of the hepatocyte and sinusoidal cell suspensions were mixed with an equal volume of 0.4% Trypan Blue dye and incubated at 37°C for 3 min. Cells were counted with a haemocytometer. The yield of purified hepatocytes was $(248 \pm 54) \times 10^6$ cells/liver (mean $\pm$ S.D.)

($n = 50$), with a viability of $86 \pm 4\%$. Sinusoidal cells numbered $(23 \pm 7) \times 10^6$ cells/liver (mean $\pm$ S.D.) ($n = 50$) with a viability of 100%. Microscopic examination revealed less than 1% cross-contamination of the hepatocytes and sinusoidal-cell preparations.

Enzyme infusion and tissue sampling. Infusion of glucocerebrosidase was carried out in rats anaesthetized with Diabital (sodium pentobarbital; Diamond Laboratories, Inc., Des Moines, IA 50304, U.S.A.) by cannulation of the femoral vein. The normal body temperature of the animals (38°C) was maintained throughout the experiment with water-circulating heating pads and monitored with a rectal probe (Aquamatic Thermia, Gormon Rupp Industries Division, Bellville, OH, U.S.A.). Blood samples were drawn from the tail vein and collected in haematocrit tubes. Samples of liver (50 mg) were cut from the periphery of lobes, weighed and immediately frozen in small glass homogenizing tubes. The fate of infused enzyme was monitored either by measuring catalytic activity above endogenous values or by measuring the ^3H or ^{125}I used in the preparation of labelled enzyme. Endogenous glucocerebrosidase activity was determined in one of two ways depending on the type of uptake being monitored. When uptake of enzyme by whole liver tissue was studied, biopsy samples were taken before the infusion of enzyme. When enzyme uptake was monitored for specific cell populations, enzymic activities were periodically determined in isolated hepatocyte and sinusoidal cell preparations from control animals infused with saline. ^3H -labelled blood and tissue samples were measured by liquid-scintillation counting with Protosol and Biofluor (New England Nuclear).

Enzyme and protein assays. Glucocerebrosidase activity was measured with either the natural

substrate D-[1- ^{14}C]glucocerebroside (Pentchev *et al.*, 1978a) or with the fluorogenic substrate 4-methylumbelliferyl β -D-glucoside (Dale *et al.*, 1979). The specific activity of purified glucocerebrosidase with the fluorogenic substrate is 70% of that found with the natural glycolipid substrate. All enzymic assays reported in the Results section were made with glucocerebroside as substrate. The fluorogenic substrate was used to determine the catalytic specific activity of the purified radioactively labelled enzyme preparations. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the hydrolysis of 1 nmol of substrate/h at 37°C . Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. Cell pellets or tissue samples were homogenized in small glass homogenizers with 10 vol. of 50 mM-citrate/phosphate buffer, pH 6.0, containing Cutscum (Fisher Scientific) (2 mg/ml) and sodium taurocholate (10 mg/ml). Samples of the total homogenate were used for the assay of glucocerebrosidase activity. Serum samples were used for the assay of enzyme activity in blood.

Results

Clearance of infused enzyme from plasma

The majority of exogenous native glucocerebrosidase was cleared from the circulation of rats with a half-life of 11 min (Table 1). Over a dose range of 5×10^4 – 2.5×10^6 units ($5 \mu\text{g}$ – 2.5 mg of protein) the quantity of enzyme infused did not appear to influence the rate at which it was cleared from the blood (Table 1). Isolation of the liver from the general circulation by ligation of the portal vein and hepatic artery increased the half-life of the enzyme in the circulation 5–6-fold.

Table 1. Plasma clearance rate of infused exogenous glucocerebrosidase

The enzyme clearance rates with various doses of intravenously infused glucocerebrosidase were measured in rats as described. The hepatic endocytic index for glucocerebrosidase uptake was calculated as described by Sinke *et al.* (1979). The fluid endocytic index was reported by Sinke *et al.* (1979) for the passive uptake of Triton and poly(vinylpyrrolidone). Results are expressed as means for the numbers of experiments indicated in parentheses.

Glucocerebrosidase infused (units)	$t_{1/2}$ of plasma clearance (min)
2×10^3 (2)	21
1×10^4 (2)	16
5×10^4 (3)	10
1×10^5 (5)	11
5×10^5 (10)	11
1×10^6 (2)	11
2.5×10 (1)	11
1×10^5 + ligation of portal vein and hepatic artery	60
Endocytic index for hepatic glucocerebrosidase uptake	$217 \mu\text{l}/\text{min}$
Hepatic fluid endocytic index	$1.1 \mu\text{l}/\text{min}$

Tissue uptake of infused enzyme

At the time of maximum hepatic uptake of infused glucocerebrosidase (15 min), the liver contained 40% of the infused dose in catalytically active form (Table 2). To examine in more detail the tissue distribution of enzyme cleared from the circulation, radioactivity was determined in various tissues 20 min and 2 h after the infusion of ^{125}I - or ^3H -labelled glucocerebrosidase (Table 2). The liver was the major depot of the exogenous enzyme. Low amounts of radioactivity were found in spleen, kidney and bone; only traces were detected in other tissues.

Distribution of exogenous enzyme in hepatic cells

Since a majority of the infused enzyme could be located in the liver, a detailed study was undertaken to characterize the cellular distribution, uptake and turnover of exogenous enzyme in this organ. A determination in six rats of the distribution of exogenous glucocerebrosidase between the total hepatocyte and sinusoidal cell population of rat liver revealed that at the time (15 min) of maximum uptake of catalytic active enzyme, 38% of the infused dose was located in hepatocytes and only 2% in sinusoidal cells (Table 3). The rate of uptake and turnover of the exogenous enzyme in the two cell populations was very similar. This distribution of exogenous glucocerebrosidase was based on cell populations that were rigorously controlled for their viability and purity. The cellular distribution of

infused asialofetuin, a specific ligand for hepatocyte receptors (Ashwell & Morell, 1974; Steer & Clarenburg, 1979) showed that the potential contamination of the sinusoidal cell preparations with hepatocytes was less than 0.4% when based on whole liver tissue (Table 4). The actual location of exogenous glucocerebrosidase in hepatocytes was confirmed by a biliary excretion over a 5 h period of 50% of the ^{125}I from infused ^{125}I -labelled enzyme (Fig. 2) and the loss of glucocerebrosidase activity from isolated hepatocyte preparations incubated with Pronase (Table 4), a treatment that specifically destroys hepatocytes, leaving sinusoidal cells intact. From the actual quantity of enzyme found in the isolated cell preparations, a quantity of exogenous glucocerebrosidase taken up by whole liver could be calculated (40%), which agreed closely with the actual values determined from whole-tissue extracts (37%) (Table 2). This correlation supports the validity of the analytical and preparative data. Over a wide range of infused doses with enzyme concentrations as high as 2.5 mg, no saturation in the hepatic uptake of exogenous enzyme could be observed with either total liver tissue or with the separate cell populations (Table 5).

Specific blockage in the hepatic uptake of infused enzyme

A series of proteins known to be taken up by hepatocytes and sinusoidal cells by carbohydrate-dependent adsorptive endocytic processes or by

Table 2. *Distribution of infused glucocerebrosidase preparations*

Native enzyme (5×10^5 units), ^{125}I -labelled (10.6×10^6 d.p.m./ $17.5 \mu\text{g}$ of protein) and ^3H -labelled (2.6×10^6 d.p.m./ $170 \mu\text{g}$ of protein) glucocerebrosidase was injected intravenously in rats. Bone samples consisted of the femoral bone and total bone radioactivity was based on bone being 10% of body weight. Muscle mass and skin were estimated to be 41% and 18% respectively of body weight (Donaldson, 1924). ^{125}I -labelled glucocerebrosidase was incubated with alkaline phosphatase as described by Fisher *et al.* (1980). The times in parentheses indicate the uptake intervals *in vivo* after infusion but before sampling.

Sample	Enzyme activity (units)			
	Native enzyme (15 min)	^{125}I -labelled enzyme (20 min)	^{125}I -Labelled enzyme treated with alkaline phosphatase (20 min)	^3H -labelled enzyme (120 min)
Liver	40	37	35	57
Spleen	—	1	—	2
Kidney	—	7	—	4
Intestine	—	1.9	—	0.3
Lung	—	1	—	0.2
Brain	—	—	—	0
Muscle	—	—	—	0
Skin	—	—	—	0.8
Bone	—	—	—	3
Blood	40	36	—	7
Urine	—	—	—	5
Bile	—	2	—	0.3
Total	80	86	35	80

Table 3. *Uptake and turnover of exogenous glucocerebrosidase in rat liver*

A dose of glucocerebrosidase (5×10^5 units) was intravenously infused in rats. At the indicated times after infusion the livers were flushed free of blood and perfused for the preparation of cells as described in the Experimental section. Enzymic activity above endogenous values was measured as described in the Experimental section and represents exogenous glucocerebrosidase uptake. The percentage of the infused dose in the total hepatocyte and sinusoidal-cell population of the whole liver was calculated using the total weight of the liver and literature values for the number of cells. Whole-liver uptake was based on the weight of the tissue sample homogenized and the weight of liver. Results are expressed as means with the numbers of experiments used to calculate the means given in parentheses.

Post-infusion interval (min)	Whole-liver content (% of infused dose in total organ)	Cellular distribution (% of infused dose in total cell population)	
		Hepatocytes	Sinusoidal cells
5 (2)	8	7	0.8
7 (1)	20	22	1.9
15 (6)	37	38	2.5
45 (2)	25	24	1.0
90 (2)	22	20	0.8

Table 4. *Characterization of the perfused rat liver system*

Exogenous glucocerebrosidase activities represent total enzymic activity recovered after infusion and uptake of 5×10^5 units of enzyme for 15 min minus endogenous activity determined in a series of control livers. Proteolytic sensitivity of the cellular location of the exogenous enzyme was carried out by incubating isolated cells with gassed buffer and 2.0% (w/v) Pronase for 45 min at 37°C followed by assay of enzymic activity in cells pelleted at 500 g for 10 min. Bolton–Hunter radioiodinated asialofetuin (50×10^6 d.p.m./0.6 mg of protein) and horseradish peroxidase (44×10^6 d.p.m./1.0 mg of protein) were intravenously infused and after uptake for 5 min the liver was prepared for perfusion as described under 'Methods'. Bile was collected on ice for 6 h. Biliary radioactivity was >90% trichloroacetic acid-soluble and cellular radioactivity was >90% trichloroacetic acid-precipitable. Cell populations represent the published values of Daust (1958) and Gates *et al.* (1961). Results are means $\pm$ s.d. and numbers of experiments are given in parentheses.

Parameter	Whole-liver tissue	Biliary excretion	Cellular distribution	
			Hepatocytes	Sinusoidal cells
Number of cells/1 g of tissue	—	—	125×10^6	65×10^6
Protein (mg/ 10^6 cells)	—	—	1.88 ± 0.2 (50)	0.05 ± 0.01 (50)
Endogenous glucocerebrosidase content of cells (units/ 10^6 cells)	—	—	126 ± 6.0 (10)	2.5 ± 1.0 (10)
Exogenous glucocerebrosidase content of cells (change in units/ 10^6 cells)	—	—	180 ± 10 (10)	17 ± 3 (10)
Exogenous glucocerebrosidase content of cells following Pronase treatment (change in units/ 10^6 cells)	—	—	28 (1)	15 (11)
125 I-labelled asialofetuin uptake (% of infused dose or d.p.m./ 10^6 cells)	80% (2)	—	7300 d.p.m. (2)	50 d.p.m. (2)
125 I-labelled asialofetuin uptake after prior infusion of 5 mg of horseradish peroxidase (% of infused dose)	83% (1)	—	—	—
125 I-labelled horseradish peroxidase uptake (% of infused dose or d.p.m./ 10^6 cells)	25% (2)	20% (1)	4700 d.p.m. (2)	1000 d.p.m. (2)

phagocytosis were tested as potential blocking agents of the clearance of exogenous glucocerebrosidase (Table 6). Asialofetuin and human lactoferrin, which have been shown to interact with specific galactose (Ashwell & Morell, 1974; Steer & Clarenburg, 1979) and fucose (Priels *et al.*, 1978) receptors respectively, did not significantly alter the uptake of glucocerebrosidase by either hepatocytes or sinusoidal cells. Heat-denatured albumin, cleared

through phagocytosis by the sinusoidal cells of the liver (Benacerraf *et al.*, 1952), similarly did not block uptake of the exogenous enzyme. On the other hand a series of glycoproteins rich in high-mannose-type oligosaccharides effectively inhibited enzyme uptake by the liver. This blockage of cellular uptake was observed not only with sinusoidal cells, but surprisingly also with hepatocytes. A more detailed study of this blockage shown in Table 7 demon-

Table 5. *The proportionality of exogenous glucocerebrosidase uptake by rat liver to the dose of enzyme infused*

The indicated doses of glucocerebrosidase (1×10^6 units/mg of protein) were intravenously administered to rats. At 15 min post-infusion the livers were flushed with Krebs–Henseleit buffer equilibrated with O_2/CO_2 (19:1) and a small portion (50 mg) of an outer lobe was removed. The liver was then perfused for the preparation of cells as described in the Experimental section. The changes in units represent the exogenous glucocerebrosidase activity and were calculated as described in the legend to Table 4. Results are expressed as means for the numbers of experiments given in parentheses.

Enzyme dose infused (units)	Enzyme uptake by whole-liver tissue (change in units/g wet wt.)	Cellular distribution (change in units/ 10^6 cells)	
		Hepatocytes	Sinusoidal cells
1×10^5 (5)	0.5×10^4	40	5
5×10^5 (5)	2.0×10^4	190	12
1×10^6 (2)	4.0×10^6	450	28
2.5×10^6 (1)	9.0×10^4	—	—

Table 6. *Effect of various proteins on the uptake in vivo of exogenous glucocerebrosidase by hepatocytes and sinusoidal cells of rat liver*

The indicated amounts of test proteins were intravenously infused in rats 3 min before the infusion of 5×10^5 units of glucocerebrosidase. After uptake for 15 min the liver was prepared for the subsequent isolation of cells as previously described. Results are expressed as means for the numbers of experiments given in parentheses.

Additions to the infusion of glucocerebrosidase		Cellular content of exogenous enzyme (change in units of glucocerebrosidase/ 10^6 cells)	
		Hepatocytes	Sinusoidal cells
None	(10)	180	17
Heat-denatured albumin (33 mg)	(2)	170	16
Human lactoferrin (10 mg)	(1)	160	18
Asialofetuin (20 mg)	(2)	155	17
Ovalbumin (50 mg)	(2)	80	8
Horseradish peroxidase (5 mg)	(2)	40	3
Invertase (3 mg)	(2)	30	2
Mannan (3 mg)	(2)	45	1

Table 7. *Inhibition in vivo of exogenous glucocerebrosidase uptake into hepatocytes and sinusoidal cells with mannose-rich glycoproteins*

The uptake of 5×10^5 units of infused glucocerebrosidase was modulated by the infusion 3 min previously of various concentrations of horseradish peroxidase or mannan. Mannan was dephosphorylated by acid hydrolysis and alkaline phosphatase treatment as described by Fisher *et al.* (1980). At 15 min the liver was processed for a measurement of the cellular distribution of the exogenous enzyme taken up as previously described. Results are expressed as means for the numbers of experiments given in parentheses.

Blocking agent		Cellular content of exogenous enzyme (units of glucocerebrosidase/ 10^6 cells)	
		Hepatocytes	Sinusoidal cells
None	(10)	180	17
Horseradish peroxidase (1 mg)	(2)	57	3.6
Horseradish peroxidase (5 mg)	(2)	38	2.7
Horseradish peroxidase (7 mg)	(1)	24	0.9
Mannan (1 mg)	(2)	76	9.0
Mannan (3 mg)	(1)	43	2.2
Mannan (6 mg)	(1)	23	1.3
Dephosphorylated mannan (3 mg)	(1)	46	2.0

strated the apparent equal sensitivity of hepatocytes and sinusoidal cells to inhibition of enzyme uptake over a range of concentrations of peroxidase and

mannan. Removal of phosphate groups from mannan by acid hydrolysis and subsequent incubation with alkaline phosphatase (Fisher *et al.*, 1980) did

not affect the degree of inhibition. This blockage by the high-mannose glycoproteins was specific, since it was shown that concentrations of peroxidase that effectively block glucocerebrosidase uptake had no influence on the hepatic uptake of [125 I]asialofetuin (Table 4). In a subsequent experiment it was shown directly that 125 I-labelled peroxidase was taken up into hepatocytes as well as sinusoidal cells and that a significant portion of this infused protein was excreted in bile (Table 4).

Biphasic nature of exogenous glucocerebrosidase cleared and catabolized in the liver

In our studies of the plasma clearance of catalytically active glucocerebrosidase it was observed that the rate of clearance was not linear but rather began to decline with time. Such multiphasic clearances have been observed for other lysosomal enzymes (Stahl *et al.*, 1976). This phenomenon was subsequently investigated in more detail with radioactively labelled glucocerebrosidase. Document-

tation of the two-component nature of infused enzyme comes from the studies with [3 H]raffinose-labelled glucocerebrosidase (Fig. 1). Raffinose covalently linked to proteins has been shown to be a stable undegradable 'tag' that accumulates at the site of tissue uptake (VanZile *et al.*, 1979). Consequently when 3 H-labelled enzyme was infused into rats there was rapid hepatic accumulation of 40% of the infused radioactivity followed by a more gradual accumulation of an additional 20% over the next 4 h

Fig. 1. Blood clearance and hepatic turnover of [3 H]-raffinose-labelled glucocerebrosidase

3 H-labelled enzyme (2.6×10^6 d.p.m./ $170 \mu\text{g}$ of protein) was intravenously infused in rat. At the indicated times blood and liver biopsy samples were taken and measured for 3 H label. The body temperature of the sedated animal was maintained at 38°C for the duration of the experiment. Δ , Total 3 H label in blood; $\bullet$, total 3 H label in liver; $\circ$, trichloroacetic acid-precipitable 3 H label in liver.

Fig. 2. A comparison of the blood clearance and hepatic turnover of freshly infused glucocerebrosidase and of the infused enzyme fraction remaining in the circulation at 90 min

125 I-labelled glucocerebrosidase (340×10^6 d.p.m./ $330 \mu\text{g}$ of protein) was intravenously infused into a rat. Over a 5 h period 50% of the infused radioactivity came to be located in the bile as trichloroacetic acid-soluble material. At 90 min 1 ml of blood was drawn from the heart (1.4×10^6 d.p.m.) with a Vacutainer tube (EDTA-coated; Becton-Dickinson, Rutherford, NJ, U.S.A.) and re-infused in a second rat. The values represent percentages of the recovered trichloroacetic acid-precipitable radioactivity. $\bullet$, Freshly infused glucocerebrosidase; $\circ$, infusion of the glucocerebrosidase fraction in blood at 90 min.

(Fig. 1). Of the total hepatic label taken up, there was an initial rapid loss of trichloroacetic acid-precipitable material, followed by a markedly lower rate of catabolism. From the blood-clearance data one could calculate that 75% of the infused enzyme was rapidly cleared ($t_1 = 11$ min) and 25% slowly ($t_1 = 90$ min). Essentially this same biphasic pattern of uptake and turnover could be observed with ^{125}I -labelled enzyme (Fig. 2). To distinguish between the alternative possibilities that the heterogeneous behaviour of the enzyme reflects a physiological response to the infusion or that specific intrinsic structural characteristics of the protein are responsible for the biphasic behaviour, a screening experiment was done. ^{125}I -labelled glucocerebrosidase was infused into a rat and at 90 min 1 ml of blood was drawn from the heart and infused in a second rat (Fig. 2). The results show that the enzyme component cleared at a slower rate from the blood and subsequently by the liver was the predominant species present in the second rat.

Discussion

The majority of glucocerebrosidase infused intravenously in rats is taken up by the liver (Table 2). This organ is the principal storage site of the excess glucocerebroside that accumulates in patients with Gaucher's disease (Brady, 1978). Although no saturability in the plasma clearance or hepatic uptake of glucocerebrosidase was observed the process was never the less shown to be governed by facilitated adsorptive endocytic mechanisms since the rate of hepatic uptake of exogenous enzyme far exceeded the rate for passive fluid endocytosis (Table 1). The rapid rate and non-saturability may indicate an endocytic process involving a high number of hepatic binding sites with a low affinity for glucocerebrosidase. Galactose and fucose receptors apparently do not play a major role in this mediated uptake, since high concentrations of asialofetuin and human lactoferrin respectively did not significantly block the endocytosis of exogenous glucocerebrosidase (Table 6). Effective blockage of hepatic uptake was achieved with glycoproteins rich in mannose (Table 7). The blockage *in vivo* of exogenous enzyme uptake into hepatocytes by the high-mannose glycoproteins was not expected, since these hepatic mannose receptors have been considered a unique domain of sinusoidal cells (Achord *et al.*, 1978; Stahl *et al.*, 1978; Brown *et al.*, 1978; Steer & Clarenburg, 1979). A lack of mannose-binding sites on hepatocytes, coupled with a blockage *in vivo* by mannose ligands of the uptake of a glycoprotein by hepatocytes as well as by sinusoidal cells, might be explained by a transfer or shuttle mechanism in which enzyme is first taken into sinusoidal cells via their mannose receptors and

subsequently transferred to hepatocytes by a second independent process. Accumulation of endocytosed material first in sinusoidal cells and subsequently in hepatocytes has been observed with carcino-embryonic antigen (Thomas *et al.*, 1977) and alkaline phosphatase (Koudstaal *et al.*, 1977). Such transfer mechanisms have also been suggested for endogenous hepatic lipids (Glauman, 1975). With regard to glucocerebrosidase uptake by liver, however, the data failed to demonstrate an intercellular transfer between the hepatocytes and sinusoidal cells. Over a 90 min period from the time of rapid uptake through rapid turnover, the ratio of exogenous enzyme in hepatocytes and sinusoidal cells remained essentially constant (Table 3). This tentatively suggested that the mannose-rich glycoproteins exerted their competitive blockage by directly interacting with hepatocytes. This interaction was strongly supported by the extensive uptake *in vivo* of ^{125}I -labelled horseradish peroxidase by hepatocytes (Table 4). Histochemical studies have also shown incorporation of peroxidase into hepatocytes (Graham *et al.*, 1969). Further, investigations of a possible common receptor that recognizes both mannose-rich glycoproteins and glucocerebrosidase will require studies with isolated hepatocyte preparations. The present findings are, however, consistent with earlier studies that reported that mannose/*N*-acetylglucosamine-binding sites on cultured rat hepatocytes appeared to play a role in the endocytosis of lysosomal α -*N*-acetylglucosaminidase (Ullrich *et al.*, 1979). Possibly the lack of uptake of certain mannose terminal glycoproteins by hepatocytes but not by sinusoidal cells may reflect, in the former but not in the latter cells, a multivalent attachment of ligand to receptors as a prerequisite for endocytic uptake. Such multivalent binding to receptors has been evoked by Fisher *et al.* (1980) for effective adsorptive endocytosis of glycoproteins.

Although a high percentage of infused glucocerebrosidase was taken up by the liver, the cellular distribution does not point to an efficient location of the exogenous enzyme for purposes of inducing glycolipid clearance in Gaucher's disease by enzyme supplementation. The very high concentration of glycolipid that accumulates in Gaucher's-disease livers does so primarily in engorged Kupffer cells (Gaucher cells) with little or no involvement of hepatocytes (Peters *et al.*, 1977). However, only 2% of the exogenous dose of glucocerebrosidase becomes located in an enzymically active form within the sinusoidal cell population (Fig. 1). Although the actual relative activities of exogenous glucocerebrosidase within the heterogeneous sinusoidal cell population are not known, if one considers that two-thirds of the sinusoidal cells are primarily made up of endothelial cells and only

one-third Kupffer cells (Zahlten *et al.*, 1978), the actual delivery of enzyme to the primary storage may be less than 1%. If one further takes into account recent radiographic studies that show that endothelial cells are from two to six times more active than Kupffer cells in the uptake of glycoproteins via the mannose receptors (Hubbard *et al.*, 1979) it becomes apparent that the effective delivery of exogenous glucocerebrosidase to the primary target cells may be precariously low. A more effective targeting to sinusoidal cells has been achieved in part by modifying the oligosaccharide side chains of the native enzyme. By exposing additional terminal *N*-acetylglucosamine and mannose residues, a 4–6-fold higher incorporation of glucocerebrosidase into sinusoidal cells was achieved (Steer *et al.*, 1978).

The rapid hepatic turnover of the exogenous enzyme points to a major area of concern. Half of the exogenous enzyme of the rat liver was extremely labile with a half-life of less than 1 h. The remaining exogenous hepatic glucocerebrosidase shows greater stability with a half-life of 12–14 h (Figs. 1 and 2). In comparison the half-life of endogenous glucocerebrosidase of mouse liver has been estimated to be 3–4 days (Hara & Radin, 1979). A similar rapid catabolic profile *in vivo* for infused human glucocerebrosidase was observed in Rhesus monkeys (Pentchev *et al.*, 1978b) and in clinical replacement trials in Gaucher's-disease patients (P. G. Pentchev, J. A. Barranger & R. O. Brady, unpublished work). A modification of the delivery system to achieve better targeting and stability of infused glucocerebrosidase was attempted in clinical trials by an incorporation of the exogenous enzyme in liposomes (Belchetz *et al.*, 1977) or erythrocytes (Beutler *et al.*, 1977) before infusion. In future studies it will be of interest to characterize both the structural features and the endocytic properties of the exogenous glucocerebrosidase fraction, which was found in our final studies to show an extended half-life in the liver (Figs. 1 and 2). This small stable portion of the total enzyme dose may account for most of the promising results observed in clinical trials of enzyme-replacement therapy in Gaucher's disease (Brady *et al.*, 1975).

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